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Preparation and antibacterial activity of Schiff bases from *O*-carboxymethyl chitosan and *para*-substituted benzaldehydes

Xueqiong Yin · Junhua Chen · Wen Yuan · Qiang Lin · Li Ji · Fang Liu

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Abstract Seven Schiff bases were synthesized from *O*-carboxymethyl chitosan (CMC) and *para*-substituted benzaldehydes. The Schiff bases were characterized through Fourier Transform Infrared Spectroscopy, Carbon-13 Nuclear Magnetic Resonance (^{13}C NMR), Distortionless Enhancement of Polarization Transfer (DEPT) 135 NMR, elemental analysis, and acid–base titration. Antibacterial activities of the Schiff bases against *Escherichia coli* (*E. coli*, ATCC 35218) and *Staphylococcus aureus* (*S. aureus*, ATCC 25923) were measured through the optical density method. Antibacterial activity of the Schiff bases differs from the substituent at the *para* position of benzaldehyde, and decreases as the sequence $OCH_3 > CH_3 > H > F > Cl > Br > NO_2$. The IC_{50} of the Schiff base from 4-methoxylbenzylaldehyde against *E. coli and S. aureus* is 30 and 34 ppm, respectively, much lower than that of chitosan (53, 48 ppm) and CMC (58, 60 ppm).

Keywords Schiff base · *O*-carboxymethyl chitosan · Synthesis · Antibacterial activity

Introduction

Chitin is the second most abundant natural polysaccharide next to cellulose, which exists in crustaceans, mollusks, insects, and fungi. Chitosan is the *N*-deacetylated

X. Yin (✉) · J. Chen · W. Yuan · Q. Lin · L. Ji · F. Liu
Hainan Provincial Fine Chemical Engineering Research Center, Hainan University, Haikou 570228, Hainan, China
e-mail: yxq88@hotmail.com

Q. Lin
Key Laboratory of Tropical Medicinal Plant Chemistry Ministry of Education, Hainan Normal University, Haikou 571158, Hainan, China

derivative of chitin, which has good physicochemical and biochemical properties, such as good biocompatibility, biodegradability, antibacterial and antifungal activities, moisture absorption, blood clotting, etc. [1–4]. Chitosan is widely investigated and used in food, cosmetics, pharmaceutical, agricultural chemicals, and many other fields [5–7].

Antibacterial activity is one of the most important bioactivities of chitosan. Chitosan expresses good antibacterial activities against many kinds of bacteria, such as *Agrobacterium tumefaciens, Corynebacterium michiganense, Escherichia coli, Micrococcus luteus, Staphylococcus aureus* [8, 9]. The antibacterial activity of chitosan is related to the amount of adsorbed chitosan on the bacterial cells. Therefore, the surface characteristics of cell walls, pH, molecular weight, and structure of chitosan are important factors for the interactions between chitosan and the cells. Higher hydrophilicity and more positively charged cell surface enable more chitosan adsorb on bacteria cells, which results in better antibacterial activity [10]. In order to increase adsorption on cell surface, chitosan is modified to change its molecular structure, and increase its solubility or positive charge [11, 12]. Low molecular weight chitosan [13] and some chitosan derivatives express better antibacterial activities than the original chitosan. Water soluble chitosan derivatives with quaternary ammonium salt express good antibacterial activities against *S. aureus,* and the activities increase with increase in the chain length of the alkyl substituent [14]. The antibacterial activities of chitosan and carboxymethyl chitosan against *E. coli* increase in the order of *N*,*O*-carboxymethylated chitosan, chitosan, and *O*-carboxymethylated chitosan [15]. Acrylic acid sodium salt (AASS) and methylacrylic acid sodium salt (MAASS) were grafted onto carboxymethyl chitosan sodium (CMCTS) to obtain copolymers (CMCTS–AASS, MCTS–MAASS) with good water solubility. Both copolymers express good antibacterial activities against *S. aureus* and *E. coli.* And CMCTS-*g*-MAASS is more effective than CMCTS–AASS [16]. Chitosan metal complexes with bivalent metal ions, including Cu(II), Zn(II), Fe(II) have much better in vitro antimicrobial activities than free chitosan and metal salts against bacteria (*S. aureus*, *S. epidermidis*, *E. coli*, *P. aeruginosa*) and fungi (*C. albicans* and *C. parapsilosis*) [17].

Schiff bases, characterized by the –N=CH– (imine) group, are active against a wide range of organisms, including bacteria, fungi, and even algaes [18–21]. $-NH_2$ of chitosan is easy to react with aldehydes or ketones to form Schiff bases. Chitosan Schiff bases are widely reported in the field of biocides [22, 23]. And many chitosan Schiff bases derivatives also express good antibacterial activity. Schiff bases of carboxymethyl chitosan (2-(2-hydroxybenzylideneamino)-6-carboxymethyl chitosan) and 2-(5-chloro-2-hydroxybenzylideneamino)-6-carboxymethyl chitosan) have better antifungal activities against Fungi *Fusarium oxysporum* f. sp. *vasinfectum*, *Alternaria solani*, and *Valsa mali* than chitosan and 6-carboxymethyl chitosan, whereas the antifungal activity of 2-hydroxybenzylideneamino-6-carboxymethyl chitosan has no difference from that of chitosan and 6-carboxymethyl chitosan [24]. The antimicrobial properties of chitosan derivatives-treated cotton fabric against *S. aureus* and *E. coli* decrease as the sequence of *O*-quaternized-*N*,*N*-biethyl-*N*-benzyl ammonium chitosans chloride > *O*-quaternized-*N*-benzylidene-chitosan > *O*-quaternized-*N*-benzyl-chitosans [25].

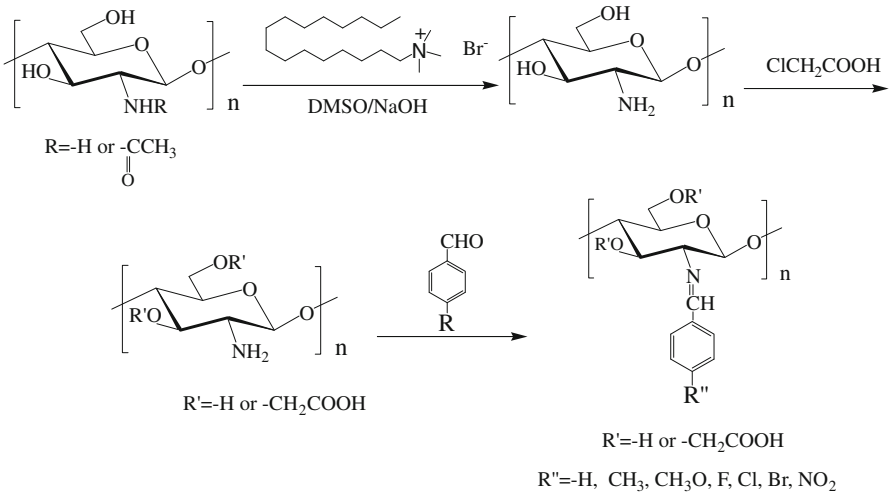

Fig. 1 Reaction scheme for the preparation of Schiff bases from chitosan

In this article, seven Schiff bases were synthesized from *O*-carboxymethyl chitosan and *para*-substituted benzaldehydes, after deacetylation and carboxymethylation of chitosan. The reaction scheme is shown in Fig. 1. The antibacterial activities of the Schiff bases, chitosan, and *O*-carboxymethyl chitosan against *E. coli* and *S. aureus* were investigated.

Experimental

Materials

Chitosan was purchased from Yuhuan Marine Biochemistry Co., Ltd. (the degree of deacetylation 85.2%,molecular weight 970 kDa). Benzaldehyde, *p*-bromide benzaldehyde, *p*-nitro benzaldehyde, hexadecyl trimethyl ammonium bromide, and monochloroacetic acid were purchased from Sinopharm Chemical Reagent Co, Ltd. *p*-methyl benzaldehyde, *p*-methoxyl benzaldehyde, *p*-fluoride benzaldehyde, and *p*-chloride benzaldehyde were purchased from Fluka. All of the reagents were used as received.

Preparation of completely deacetylated chitosan (DCTS)

Chitosan was completely deacetylated according to the Reference [26]. 200 mL dimethyl sulfoxide (DMSO), 20 g sodium hydroxide (NaOH), and 0.4 g surfactant hexadecyl trimethyl ammonium bromide were added into a 500 mL three-neck flask. The mixture was heated until it dissolved, and then 10 g chitosan was added into the flask. After the reaction was allowed for 3 h at 130 °C under the protection of nitrogen, the reaction mixture was cooled to room temperature, filtrated, and

washed with distilled water until neutral. White DCTS was obtained after lyophilization.

Preparation of *O*-carboxymethyl chitosan (CMC)

CMC was produced as described in the Reference [27]. 2 g DCTS was put in a 100 mL beaker. Then, 20 mL of 40% NaOH was dropped into chitosan. The chitosan NaOH mixture was frozen at −18 °C overnight, and then thawed to obtain alkaline chitosan. Chitosan was transferred into a three-neck flask, and then 20 mL isopropanol was added. 9.6 g monochloroacetic acid in 20 mL isopropanol was dropped into chitosan mixture with stirring at 30 °C. The reaction was allowed for 4 h, and then diluted hydrochloric acid was used to neutralize the solution. The mixture was precipitated by ethanol, filtrated, and washed alternatively with 80% methanol and ethanol. White powder CMC was obtained after vacuum dried at 50 °C for 6 h.

Synthesis of Schiff bases from *O*-carboxymethyl chitosan (BCMC)

1 g CMC was dissolved with 100 mL water in a three-neck flask. Benzaldehyde ethanol solution was added into the flask under stirring. Schiff reaction between CMC and benzaldehyde was allowed at 65 °C for 4 h under reflux. The product was precipitated with anhydrous ethanol, washed with 80% methanol and ethanol alternatively, extracted in a Soxhlet flask with mixture of ethanol and ethyl ether (1:1/vol:vol), and then vacuum dried at 50 °C. In the end, *O*-carboxymethyl-*N*-benzylidene chitosan (H-BCMC) powder was obtained. According to the same procedure, *O*-carboxymethyl-*N*-(*p*-methoxyl-benzylidene)-chitosan (CH_3O-BCMC), *O*-carboxymethyl-*N*-(*p*-methyl-benzylidene)-chitosan (CH_3-BCMC), *O*-carboxymethyl-*N*-(*p*-fluoro-benzylidene)-chitosan (F-BCMC), *O*-carboxymethyl-*N*-(*p*-chloro-benzylidene)-chitosan (Cl-BCMC), *O*-carboxymethyl-*N*-(*p*-bromo-benzylidene)-chitosan (Br-BCMC), and *O*-carboxymethyl-*N*-(*p*-nitro-benzylidene)-chitosan (NO_2-BCMC) were prepared.

Measurement of antibacterial activities

By the optical density method as described [15], antibacterial activities of chitosan and its derivatives against *Escherichia coli* (*E. coli*, ATCC 35218) and *Staphylococcus aureus* (*S. aureus*, ATCC 25923) were evaluated. A representative colony from solid nutrient agar was picked off with a wire loop and placed in 75 mL nutrient broth (peptone 10 g, beef extract 3 g, and NaCl 3 g in distilled water 1000 mL; pH 7.0), which was then incubated in an air bath shaker at 37 °C for 24 h with the shaking speed 130 revolutions per minute (rpm). Then, a culture where bacteria grew in a logarithmic growth phase was prepared for an antibacterial test. The antibiotics were dissolved in 1% acetic acid and the pH of the solution was adjusted to 5.5 with 10% NaOH. 100 μL bacteria solution was put into a tube with 4 mL nutrient broth containing 100 μL sample solution. Bacteria were cultivated in a shaker with shaking speed 130 rpm, at 37 °C for 20 h. Then, the turbidity of the

medium was measured at 610 nm by a UV–vis spectrophotometer (Shimadzu UV-2450 UV–VIS Spectrophotometers). Deionized water was used to replace the sample solutions for blank test. Each experiment was performed three times, and the data were averaged. The standard errors of the experiments are all less than 5%.

Characterization

Fourier Transform Infrared Spectroscopy (FTIR) spectra were measured on a Bruker TENSOR27 spectrometer, using KBr pellet technique. ^{13}C NMR and DEPT 135 NMR spectra were recorded using Bruker AV 400 (400 MHz) spectrometers in 1% deuterium chloride (DCl) at room temperature (sample concentration 5–10%). The scan number was 12000 for ^{13}C NMR spectra. Elemental analysis (EA) results were obtained using a PE 2400 elemental analyzer.

Results and discussion

Characterization of chitosan and its derivatives

Figure 2 shows the FTIR spectra of chitosan (CTS, a), deacetylated chitosan (DCTS, b), CMC (c), and *O*-carboxymethyl-*N*-(*p*-CH_3O-benzylidene) chitosan (CH_3O-BCMC, d). For CTS, the peak at 1653 cm^{-1} is the characteristic absorbance of the amide-I band, and 1578 cm^{-1} is the bending vibration band of $–NH_2$. After

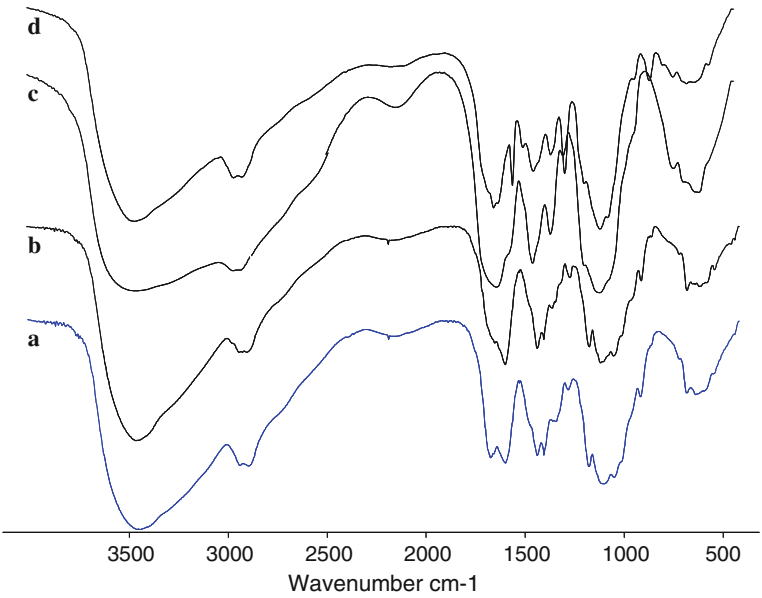

Fig. 2 FTIR spectra of chitosan (CTS, *a*), deacetylated chitosan (DCTS, *b*), CMC (*c*), and CH_3O-BCMC (*d*)

Table 1 FTIR data of Schiff bases (cm^{-1})

Samples	$\nu_{(H–O \text{ and } H–N)}$	$\nu_{(C–H)}$	$\nu_{as(–COO–)}$ and $\nu_{(arC–C)}$	$\nu_{(arC–C)}$	$\nu_{(–COO–)}$	$\nu_{(C–N)}$	$\nu_{(C–O)}$	DS (%)
H-BCMC	3423	2877	1595	1502	1407	1322	1067	51.5
CH_3O-BCMC	3423	2923	1609	1515	1409	1313	1072	53.2
CH_3-BCMC	3439	2876	1607	1515	1410	1322	1140	50.0
F-BCMC	3425	2877	1604	1512	1412	1322	1070	45.7
Cl-BCMC	3423	2877	1595	1492	1409	1321	1070	44.3
Br-BCMC	3423	2875	1591	1487	1406	1321	1070	43.5
NO_2-BCMC	3420	2879	1602	1517	1400	1347	1070	38.5

deacetylation, the absorbance of the amide-I band around 1653 cm^{-1} disappeared, which means chitosan has been deacetylated. The peak at 1070 cm^{-1} is due to –C–O asymmetric stretching vibration. Compared with the spectrum of DCTS, CMC has an obvious peak around 1595 cm^{-1}, which is resulted from the asymmetric stretching of –C=O (–COONa) [28]. A shoulder appears around 1521 cm^{-1}, which is the absorbance of NH_3^+ [29]. For *p*-CH_3O-BCMC, there are two new peaks appeared at 1610 and 1514 cm^{-1}, which are the characteristic absorbances of benzene ring [24]. The peak from asymmetric stretching of –C=O (–COONa) and the characteristic absorbance of imino groups overlap with the peak at 1610 cm^{-1}. The FTIR data of other Schiff bases were shown in Table 1. Each sample has specific absorbances: absorbance of $\nu_{(H–O \text{ and } H–N)}$ around 3420–3439 cm^{-1}, $\nu_{(C–H)}$ around 2875–2923 cm^{-1}, $\nu_{as(–COO–)}$ around 1591–1610 cm^{-1}, $\nu_{(arC–C)}$ around 1487–1517 cm^{-1}, $\nu_{(–COO–)}$ around 1400–1412 cm^{-1}, $\nu_{(C–O)}$ around 1067–1140 cm^{-1}, and $\nu_{(C–N)}$ around 1313–1347 cm^{-1}. The DS of benzylidene is shown in Table 1, which was determined by EA. The DS arranges from 38.5 to 53.2%.

Figure 3 shows the ^{13}C NMR spectrum of DCTS in 1% DCl. The respective characteristic absorbance of C-1, C-4, C-5, C-3, C-6, and C-2 appears at 97.6, 76.5, 74.9, 70.2, 60.2, and 55.9 ppm [30]. Absence of the peak of C=O around 175 ppm and the peak of methyl around 20 ppm express acetyl has almost completely been removed [31], which is in agreement with the result from FTIR.

Figure 4 shows the ^{13}C NMR spectrum (a) and DEPT 135 NMR spectrum (b) of CH_3-BCMC in 1% DCl. There is an obvious peak at 174.1 ppm assigned to –COOH of carboxymethyl group [32]. The peak at 168.7 ppm belongs to imino groups (C=N) [33]. According to the DEPT 135 NMR spectrum, there are four different $–CH_2$ in CH_3-BCMC. The shift of unsubstituted C-6 is at 60.2 ppm, while that of the substituted C-6 has shifted to 68.1 ppm because of the electron-withdrawing effect of the carboxymethyl. The other two negative peaks at 50.9 and 47.4 ppm result from methylene groups ($–CH_2$) of $–CH_2COOH$ attaching to C-6 and C-3 [32], respectively. Peaks at 140.4, 130.1, and 127.2 are assigned to C-8, C-9, 10, and C-11 on the benzene ring. Absorbance of C-12 ($–CH_3$) locates at 20.4 ppm. The peak of substituted C-2 is shifted from 55.9 to 60.8 ppm. The signal due to C-1 is split at 96.6 and 97.5 ppm because of the Schiff base reaction happened at C-2, and the

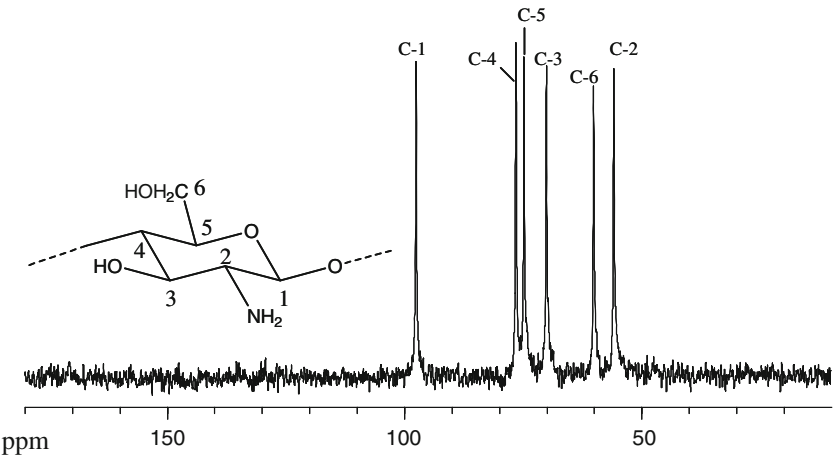

Fig. 3 ^{13}C NMR spectrum of chitosan in 1% DCl

absorbance of C-3 is also split to two peaks at 69.0 and 69.8 ppm. The signals of C-4 and C-5 remain at 76.5 and 74.9 ppm, respectively.

Antibacterial activities of chitosan and its derivatives

The antibacterial activities against *E. coli* and *S. aureus* bacteria were measured with different sample concentrations (240, 180, 120, 60, and 24 ppm) at 37 °C for 20 h, pH 5.5. The turbidity of the solutions was measured after cultivation. The plots of optical density (OD) versus concentration were shown in Figs. 5 and 6. As shown in Figs. 5 and 6, comparing with the control, all of the samples express better antibacterial activities. The antibacterial activities of the samples against both *E. coli* and *S. aureus* decrease as OCH_3-BCMC > CH_3-BCMC > H-BCMC > F-BCMC > DCTS > CMC > Cl-BCMC > Br-BCMC > NO_2-BCMC. After carboxymethylation, the antibacterial activity of CMC against both *E. coli* and *S. aureus* decreased slightly, a little lower than that of chitosan. The antibacterial activity of Schiff bases differs from the substituent of benzaldehyde. And with the increase of the concentration of DCTS, O-CMC, and BCMCs, ODs of the medium decreased. The ODs of 240 ppm CH_3-BCMC and CH_3O-BCMC against *E. coli* were 0, which revealed that *E. coli* bacteria were completely inhibited. And ODs of 180 ppm CH_3-BCMC and CH_3O-BCMC against *S. aureus* were both 0. Therefore, *S. aureus* were completely inhibited by 180 ppm CH_3-BCMC and CH_3O-BCMC.

Chitosan is well known for its antibacterial activity. Two antibacterial mechanisms of chitosan are widely accepted: (1) the positive groups $–NH_3^+$ on the backbone of chitosan polymer combine with the negatively charged bacterial surface to disturb the cell membrane and cause cell death [33]; (2) chitosan oligomer permeated the cell membrane block the transcription from DNA and interfere with the RNA and protein synthesis, which inhibit the propagation of bacteria [34]. The molecular weight of the original chitosan in this article is

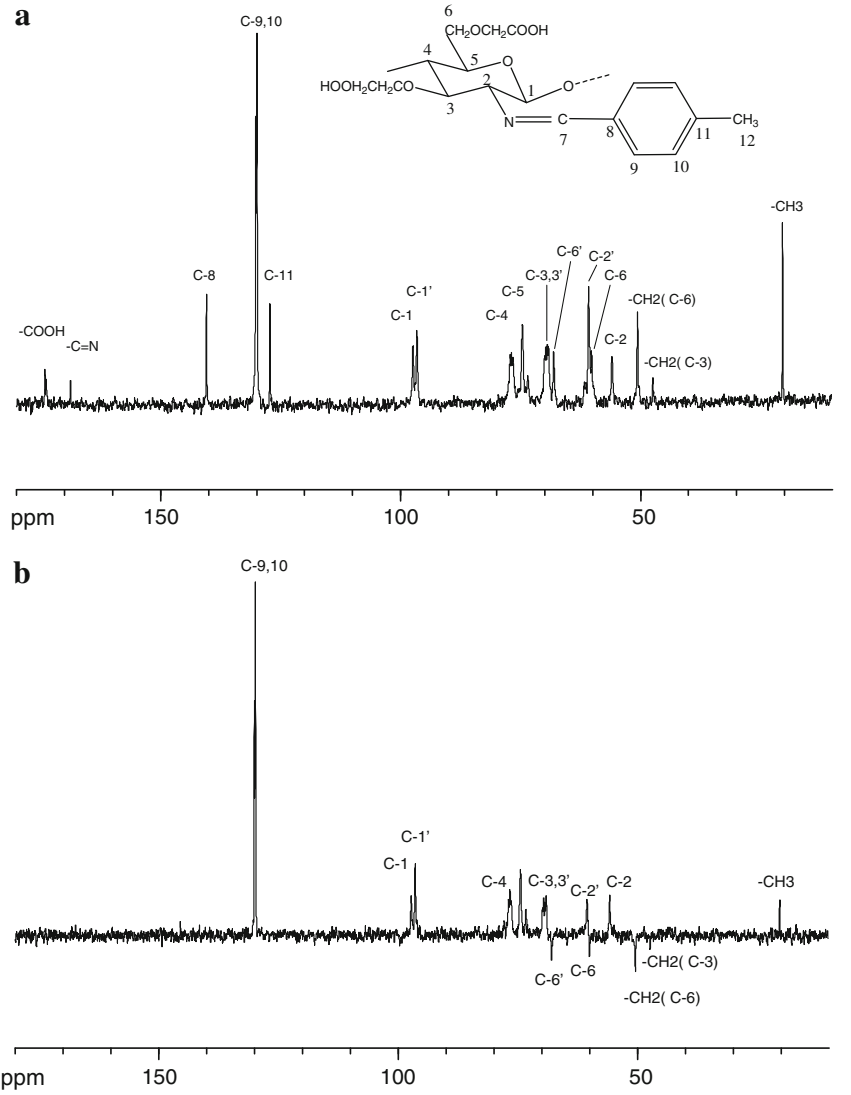

Fig. 4 ^{13}C NMR spectrum (**a**) and ^{13}C DEPT 135 NMR spectrum (**b**) of CH_3-BCMC in 1% DCl

970 kDa. It is impossible for the chitosan to permeate the cell membrane. Therefore, chitosan and its derivatives should interact with the bacteria following the first mechanism in this investigation. $–NH_2$ of chitosan should have been significantly transferred to $–NH_3^+$ in this experiment, because the antibacterial activity was measured at pH 5.5. Free H^+ from $–CH_2COOH$ competes with $–NH_3^+$ of chitosan in binding to the negatively charged bacterial surface, which results in less CMC combining with bacteria than chitosan. Because only polycationic compound can cause cell agglutination and cell death, the antibacterial activity of CMC is lower than that of the original chitosan [15].

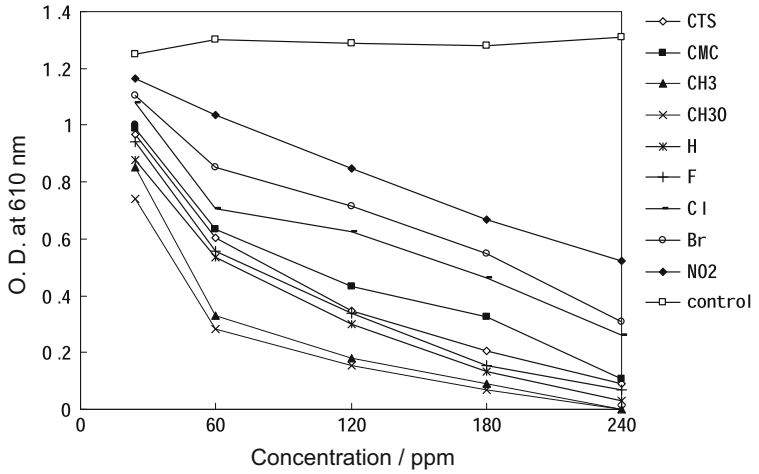

Fig. 5 Effects of concentration on the antibacterial activities of CTS and its derivatives against *E. coli*

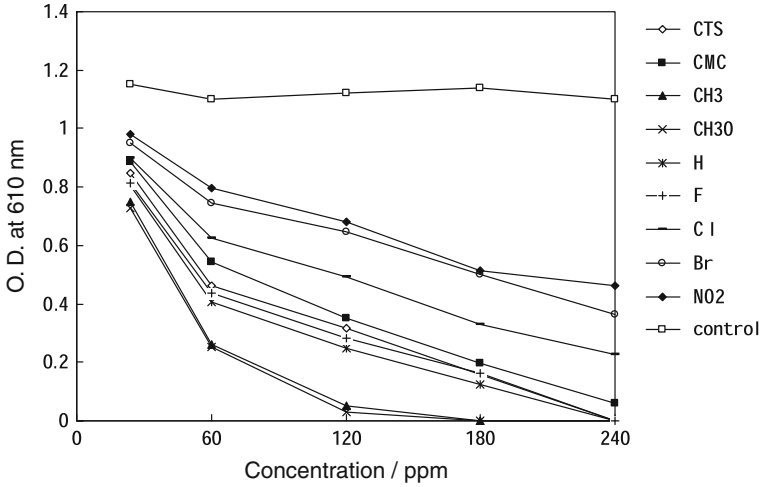

Fig. 6 Effects of concentration on the antibacterial activities of CTS and its derivatives against *S. aureus*

For both *E. coli* and *S. aureus*, H-BCMC has better antibacterial activity than CMC, which is resulted from the synergy effects of chitosan and Schiff base [24]. Charge density is important for both chitosan and Schiff bases antibiotics [35]. The substituent constant σ of the substituent of benzene ring increase as $\sigma_{OCH_3} < \sigma_{CH_3} < \sigma_H < \sigma_F < \sigma_{Cl} < \sigma_{Br} < \sigma_{NO_2}$ [36]. Because of conjugate effects and inductive effects caused by the substituents at the *para* position of benzaldehyde, the charge density of benzene cycle in Schiff bases decrease with the increase of σ value [37]. And as shown in Table 2, the antibacterial activities of the seven Schiff bases decrease accordingly. The IC_{50} of CH_3O-BCMC, NO_2-BCMC against *E. coli* and *S. aureus* are quite different, being 30, 194 ppm and 34, 158 ppm, respectively.

Table 2 Structure–antibacterial activity relationship of chitosan and its derivatives

Samples	Substituent constant (σ)	*E. coli* (OD)	IC_{50} (ppm)	*S. aureus* (OD)	IC_{50} (ppm)
CH_3O-BCMC	−0.268	0.153	30	0.032	34
CH_3-BCMC	−0.170	0.181	35	0.051	36
H-BCMC	0	0.301	47	0.247	42
F-BCMC	0.062	0.337	49	0.282	46
Cl-BCMC	0.227	0.626	79	0.491	89
Br-BCMC	0.232	0.714	153	0.648	155
NO_2-BCMC	0.778	0.846	194	0.681	158
CTS		0.345	53	0.315	48
O-CMC		0.432	58	0.351	60

Comparing with –H, electron donating groups (–CH_3, –OCH_3) increase the antibacterial activities of BCMC, while electron withdrawing groups (–F, –Cl, –Br, and –NO_2) decrease the activities. The results are in agreement with those reported by Hou et al. [38], and verify that charge density is an important factor for antibacterial activities of Schiff bases. It is possible to design new CTS Schiff bases derivatives with high antibacterial activities through adjusting the charge density of the benzene ring.

Conclusion

After deacetylation and carboxymethylation of chitosan, seven Schiff bases were successfully synthesized from *O*-carboxymethyl chitosan and *para*-substituted benzaldehydes. Synergy effect of chitosan and Schiff base increases the antibacterial activities of chitosan against *E. coli* and *S. aureus*. Electron-donating group at the *para* position of benzaldehyde increases the antibacterial activities of the chitosan Schiff bases, while electron-withdrawing group decreases the antibacterial activities. The substituent type of benzaldehyde has impact on the antibacterial activities of Schiff bases. The position of the substituents may also affect bioactivities, which should be investigated further. Chemical modification, such as Schiff reaction, esterification, etherification, carboxymethylation, alkylation, etc., is a good way to improve the properties and bioactivities of chitosan. Schiff bases of chitosan are potential as antibiotics in the fields of agriculture, textile, environmental process, biomaterial, and food. Preparation and antibacterial structure–activity research of Schiff bases directly from chitosan and *para*-substituted benzaldehydes will be reported in our next article.

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